PATENT
0249-0119P

IN THE U.S. PATENT AND TRADEMARK OFFICE

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Appl. No.:	09/529,937	Group:	1615
Filed:	June 30, 2000	Examiner:	G. Kishore

For: DINITROANILINE LIPOSOMAL FORMULATIONS AND PROCESSES FOR THEIR PREPARATION

DECLARATION UNDER 37 C.F.R. §1.132

Commissioner for Patents
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Sir:

1. Declarant is one of the co-inventors of the above-identified U.S. Patent application.

2. Declarant is aware of the Final Office Action in the above-noted application and wishes to state that certain experiments were carried out to show that the invention in said application is directed to a most unexpected improvement in the treatment of infections by certain microorganisms in warm-blooded animals and humans.

3. Declarant has graduated from Wolfgang Göethe Universität of Frankfurt/Main, Germany with a Dr. phil. nat. degree in Naturwissenchaften; further Declarant has done research in the field of Drug delivery systems for over 17 years.

4. Under Declarant's direction, the experiments carried out are described as follows.

A drug delivery system needed to be developed for the use of

trifluralin by parenteral route against visceral and cutaneous forms since trifluralin solubility and lipophilicity do not allow the administration by any other route than the topical one. By this route, no activity against the visceral forms was observed in the literature.

In view of the difficulties above described, an alternative approach to finding new drugs is the drug encapsulation in macrophage directed carriers, as liposomes. Liposomes are phospholipid synthetic bilayer vesicles capable of incorporating a variety of substances independently of their molecular weight, electrical charge and solubility.

The rationale for the use of liposomal associated drugs instead of free drugs for the treatment of visceral leishmaniasis relies on the fact that amastigotes of the parasite are specifically located in liver, spleen, and bone marrow macrophages. As liposomes are preferentially taken up by these cells they can deliver toxic agents straight to the intracellular location of established parasites. Though, the administration of liposome-encapsulated agents theoretically increases the therapeutic index of the agent in two ways: 1) increasing the uptake of the carrier and consequently of the drug by macrophages contained organisms, and 2) reducing toxicity of the free drug due to relatively low uptake of carrier by organs to which the drug is toxic.

The great majority of drug delivery systems administered by

the intravenous route are taken up from circulation by the liver, meaning that they accumulate preferentially in this organ, not achieving, at significant quantities, other organs also belonging to MPS (spleen and bone marrow) The uptake by these other organs can be increased by the reduction of the vesicle diameter. This can result in the suppression of the infection in the spleen and bone marrow, quite difficult to achieve with the free drugs.

Results in literature up to the present have shown that liposome encapsulated drugs are usually much safer and more effective to treat MPS infection as compared to free drugs.

Description of Experimental Methods

Method 1

Experiments have shown the achievement, under stable form, whether lyophilized or not, of liposomal formulations containing one or several dinitroanilines, for example trifluralin, incorporated or encapsulated.

In the formulations obtained according to these experiments, the liposomal diameter varies between 0.01 μm to 50 μm. According to one of the preferred forms of preparation, mixtures of different size populations exist in the formulations of the present invention.

Additionally, the experimental formulations are envisioned

to contain any of the following lipids, hydrogenated or not, individually or in mixtures, in any molar ratio distearoylphosphatidylcholine (DSPC), phosphatidylcholine (PC) cholesterol (Chol) or derivatives; sphingomielin (SM), dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidylglycerol (DOPG), phosphatidylglycerol (PG), dimiristoylphosphatidylcholine (DMPC), dipalmitolphosphatidylcholine (DPPC), gangliosides, ceramides, phosphatidylinositol (PI), phosphatydic acid (PA), dicetylphosphate (DCP), dimiristoylphosphatidylglycerol (DMPG), stearylamine (SA), dipalmitoylphosphatidylglycerol (DPPG) and other synthetic lipids.

The preparation process of these experimental liposomal formulations comprises the steps of:

- (a) hydration from a lipid film containing the dinitroaniline for the achievement of a liposomal formulation;
- (b) lyophilization; and
- (c) rehydration.

In a common way, solubilization in organic solvent of the lipidic components and the dinitroaniline or dinitroanilines, for example trifluralin, has been achieved, followed by drying under N_2 stream or under vacuum, for example, in a rotavapor with controlled temperature for the achievement of a mixed homogeneous film of lipid and dinitroaniline or dinitroanilines, for example, trifluralin. This film has been subsequently, hydrated with a sugar solution forming multilamellar liposomes. The following

step has achieved the liposomal formulation sizing, under pressure, by successive extrusions through polycarbonate membranes of pore sizes varying from 5.0 to 0.05 μm. The sizing has ended preferably after extrusion through the membrane with the desired pore size for a part of the population. After the attainment of the necessary different populations with well-determined diameters, the following step is the mixture of these populations.

After the attainment of the necessary different populations of well-determined diameter, the following step is the mixture of these populations. After the mixture of the populations, a concentrative dialysis is done using, for example, polyethyleneglycol as hygroscopic agent, followed by a step of dehydration. This dehydration occurs in the presence of sugars that act as protective against sublimation of the dinitroaniline or dinitroanilines, for example, trifluralin.

The formulations so obtained, after hydration with water, are ready for use in administration to animals or humans, e.g. parenterally.

According to these experiments, in order to prepare the multilamellar liposomes a step of drying a mixture of one dinitroaniline, namely trifluralin, and lipids, both solubilized in the same solvent or mixture of organic solvents, is performed. The amount of trifluralin varies according to the final volume to prepare, ranging from 10 μg to 1 g or more. The amount of lipid

also changes according to the final volume to be prepared, ranging from 1 μmole to 1 mole or more. The adequate lipids, hydrogenated or not, for the preparation of the formulations are present individually or in mixtures, in any molar ratio from the following lipids: distearoylphosphatidylcholine (DSPC), phosphatidylcholine (PC), cholesterol (Chol) or derivatives, sphingomielin (SM), dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidylglycerol (DOPG), phosphatidylglycerol (PG), dimiristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine (DPPC), gangliosides, ceramides, phosphatidylinositol (PI), phosphatydic acid (PA), dicetylphosphate (DcP), dimiristoylphosphatidylglycerol (DMPG), stearylamine (SA), dipalmitoylphosphatidylglycerol (DPPG) and other synthetic lipids.

The sole obtained mixture is submitted to a step of drying under a N_2 stream, until the complete removal of the solvent or mixture of solvents. After drying, hydration of the mixture with a solution of a sugar as, for example, trehalose, is done, ranging in concentration from 0.01 M to 2 M, under mechanical stirring or manual external stirring. The so obtained liposomal formulation is, then, submitted to a step of sizing, by successive passages under pressure through polycarbonate filters of decreasing pore diameter, referred to as extrusion. Extrusion starts normally through 5 μm diameter pore membranes and continues with passages through diameter pore membranes of 2, 1,

0.8, 0.6, 0.4, 0.2, 0.1 and 0.05 μm, reaching sometimes 0.02 μm. After passage through 0.4 μm membranes, the so obtained liposomal preparation is split into two parts. Only one of those parts goes through the rest of the extrusion procedure until, for example, 0.05 μm diameter pore membranes. At the end, the two parts that correspond to two distinct populations of liposomes are mixed, achieving, by this way, one liposomal formulation containing liposomes that exhibit two different diameter distribution populations. The simultaneous presence of these different diameter populations present the surprising advantage that, after *in vivo* parenteral administration, the population of bigger diameter is rapidly captured by mononuclear phagocytic system cells, while the small size population remains in circulation, reaching organs other than liver and spleen, where the parasitic infection also exists, as for example, the bone marrow.

The so obtained formulation has been submitted to a step of concentration by dialysis against, for example, polyethyleneglycol that acts as a water removing agent. After this dialysis step, the formulation is frozen down to -70°C during, at least one hour, after which it is submitted to lyophilization.

After this lyophilization, the formulation is ready to be used, after the addition of water to the so obtained powder. Hydration occurs instantaneously originating one homogeneous suspension of liposomes in water, containing the dinitroaniline

or dinitroanilines as, for example, trifluralin.

Method 2

In a particularly preferred way of preparation of the liposomal formulation, a lipid mixture of DOPC:DOPG in a molar ratio of 7:3, in a total of 10 μmole of lipid, solubilized in chloroform, is mixed with 1 μmole of trifluralin, solubilized in chloroform. The obtained mixture is, then, dried under a stream of nitrogen until total evaporation of the chloroform. The resulting film is hydrated with 0.1 mL of 0.3 M trehalose, with manual stirring. After complete resuspension of the lipidic film, the formulation rests for 15 minutes, after which 0.1 mL more of the same trehalose solution is added. Another 15 minutes resting period is allowed after which hydration is completed by adding 0.8 mL of the same solution. The so obtained liposomal formulation is submitted to a sizing step by passage under pressure through polycarbonate membranes of successively decreasing pore diameters, from 5 μm to 0.4 μm. After this extrusion procedure, the formulation is divided in two equal parts. One of those parts continues the sizing step up to a filter of 0.05 μm. The two populations so obtained are, then, mixed. The mixed liposomal formulation is submitted to freezing at -70°C for 60 minutes and, after that period, lyophilized. In this way, a liposomal formulation ready to be hydrated with 1.0 mL of distilled sterile water is obtained, which is able to be

parenterically administered.

The formulations may also contain auxiliary substances, pharmaceutically acceptable, and useful for preservation of their quality, and/or to adjust them closely to related physiological conditions, such as pH adjusting agents, buffering agents, tonicity agents, antioxidants and other adjuvants as, for example, sodium acetate, sodium lactate, sodium chloride, potassium, chloride, calcium chloride, glucose, saccharose, mannitol, xylitol, alpha-tocopherol.

The pharmaceutical formulations obtained according to the present experiments are administered to warm blooded animals, such as man, already suffering from leishmaniasis, during the necessary time interval and in a necessary quantity to end or significantly inhibit infection progress. The adequate quantities for the achievement of that effect are named as ''therapeutically efficient doses''. The therapeutic efficient doses for this use will depend on the infection degree and on the general state of health of the treatment individual. There is no other formulation of the free drug, namely, trifluralin, used in parenteric administration.

The following examples, of liposomal formulations prepared according to these methods and of their respective physico-chemical and biological analysis,are presented as illustrations below.

Trfluralin (TFL) Incorporation in Liposomes

The preparation of the following described formulations, in a total volume of 5 mL for lipidic composition, started by the addition of TFL to lipid in chloroform, followed by evaporation of the solvent under a nitrogen stream. Hydration of the resulting film was done by adding 500 μL of trehalose 0.3 M, stirring and resting for 15 minutes, addition of 500 μL more of 0.3 M trehalose, stirring again and resting again for a new 15 minutes period and, finally, by the addition of 4000 μL of 0.3 M trehalose. Samples for dosage (initial TFL and initial lipid) were removed. The liposomal formulations so obtained were sized by successive filtration, under nitrogen pressure, through polycarbonate filters with pores of 5.0, 2.0, 1.0, 0.8, 0.6 and 0.4 μm, with two passages in the last filter (extrusion). The non-incorporated TFL, as it is insoluble in aqueous solutions, crystallizes as a needle type structure and remains at the top of the filters. After extrusion through 0.4 μm filter, the formulations are split into two equal parts. With one of those parts, the extrusion procedure continues, now through diameter pore membranes of 0.2 and 0.1 μm, with two passages in the last filter. The liposomal formulation half part that was extruded until membranes of 0.4 μm pore diameter, is named VET400 (Vesicles Extruded Through 400 nm). The liposomal formulation half part that was extruded until membranes *of* 0.1 μm port

diameter is named VET100 (Vesicles Extruded Through 100 nm). The VET 400 and VET 100 formulations obtained by the previous process are finally submitted to dosage (final TFL and final LIP).

Single Dose Toxicity Evaluation

This study was performed with a liposomal formulation of TFL with DOPC:DOPG 7:3 as the lipid composition and compared with a liposomal formulation with equal lipid composition without TFL (empty liposomes).

The preparation of the empty liposomal formulation for this single dose toxicity study, 400 mL initial volume containing 10 μmole/mL of lipid (DOPC:DOPG) in 7:3 molar ratio, started by measuring of lipid in chloroform, followed by evaporation of the solvent under a nitrogen stream. The hydration of the resulting film was carried out by addition of 40 mL of 0.3 M sucrose, stirring 30 minutes rest, addition of 40 mL more of 0.3 M sucrose, stirring again and resting again for 15 minutes more and, finally, with the addition of 320 mL of 0.3 M sucrose.

The so obtained empty liposomal formulation was sized by successive filtration under nitrogen pressure, through polycarbonate filters with pores of 5.0, 2.0, 1.0, 0.8, 0.6 and 0.4 μm, with two passages in this last filter (extrusion). After extrusion the liposomal formulation was submitted to ultracentrifugation at 49,000 rpm, for two hours, at 15°C. After

ultracentrifugation, supernatant was removed and the pellet resuspended up to 35 mL by addition of 0.3 M sucrose.

The preparation of the TFL liposomal formulation was performed according to the same process described for the empty formulation, with TFL being added to the initial solution of lipid in chloroform. After extrusion through 0.4 μm filter, the formulations are split in two equal parts. With one of those parts extrusion procedure continues, now through diameter pore membranes of 0.2 and 0.1 μm, with two passages in the last filter. The TFL formulations VET400 and VET100 obtained according to the previous process were mixed, being this mixture of the two previous formulations named as MIX liposomal formulation. The formulation was microscopically observed for crystal detection that, if present, would be removed by centrifugation.

The final lipid concentration was determined in both empty and TFL liposomal formulations being the last one adjusted in a way that both formulations contained exactly the same concentration of lipid. After this adjustment the TFL concentration was determined in the TFL containing formulation.

The study was carried out in BALB/c male and female mice. The liposomal formulations were administered by two routes: intraperitoneal (i.p.) and intravenous (i.v.). The administered doses were 30, 20 and 10 mL/kg for the i.p. route and of 10, 5 and 2 mL/kg for the i.v. route of administration. Five animals per group were used. The administered doses correspond to

calculated doses of lipid (in both formulations) and of TFL (in the TFL containing formulations).

Table 2 - Single dose toxicity doses

Dose (mL/Kg)	Lipid (μmole/Kg)	TFL (mg/Kg)
30	2790	63.9
20	1860	42.6
10	930	21.3
5	465	10.7
2	186	4.3

All animals were weighed and the amount of liposomal formulation was calculated according to the measured weight in order to achieve the desired dose, in mL/kg. One animal group per sex was injected with 0.3 M sucrose as a control group.

The animals were observed at regular intervals, during 48 hours after administration, for detection of behaviour changes. After that period, animals were euthanised, weighed and, from each animal, heart, spleen, liver and kidneys were removed, weighed and observed for macroscopical changes. Relative organ weight was calculated as the ratio between the organ weight and the weight of the animal. The obtained results showed unexpected improvement in administration of trifluralin to the animals.

The obtained values were statistically treated for the

significance of variations (p=0.005). Obtained results for absolute weights (full animal and separate organs) in both formulations were compared between themselves and with absolute weight of the control group. The total absence of toxicity of any of the injected formulations was concluded from the statistical analysis.

Biological Activity Evaluation

In order to evaluate the biological activity of TFL liposomal formulations prepared according to the present invention, one animal model of leishmaniasis was selected. BALB/c mice were infected with 2 x 10^7 (i.v.) LV-9 *(Leishmania donovani)* parasites, obtained from the London School of Hygiene and Tropical Medicine. The groups (5 animals per group) and treatment schedules are presented in Table 1.

Table 1 - Biological Activity

Formulation lipidic composition	Dose (mg TFL/kg)	N^{er} of Doses	Inhibition %
DOPC:DOPG 7:3	15	5 3 1	62 17 80
DSPC:CHOL 4:1	15	5 3	53 92 91
PC:PG 4:1	15	5 3 1	47 88 60
PC:CHOL:PI *3.7:1:0,3*	5	5 3 1	57 52 68
PC:CHOL:DSPE-PEG (2000) 3.7:1:0,3	4	5 3	75 74
DOPC:DOPG 7:3 (dialysed)	0,6	5	86

Treatments started 7 days post-infection and animals were euthanised 15 days post-infection. Liver was removed and weighed from each animal and amastigote counting was performed in each one by smear impression. The infection was calculated through an appropriate mathematical equation. The so obtained results are

expressed in Table 1.

The first conclusion is that, due to the liposomal incorporation of TFL, parenteric administration of TFL is possible.

All TFL formulations were able to reduce infection in this model as compared to negative control / non treated animals. Due to absence of toxicity these formulations have therapeutic index higher than many other antileishmanial drugs.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Dated this 18th day of Dec. 2003

Mo Zeughrie Mo Lamy

Signature of Declarant